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Short communication

Lack of NMDA receptor subunit exchange alters Purkinje cell dendritic morphology in cerebellar slice cultures

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Abstract

Early postnatal developmental changes in *N*-methyl-D-aspartate (NMDA) receptor (NR) subunits regulate cerebellar granule cell maturation and potentially Purkinje cell development. We therefore investigated Purkinje cell morphology in slice cultures from mice with genetic subunit exchange from NR2C to NR2B (NR2C-2B). NR2C-2B Purkinje cells after 12 days *in vitro* showed a significantly impaired dendritic arbour complexity with reduced branching density as compared to wild-type cells, a phenotype that was reversed by NMDA treatment. These data support the concept that in cerebellar slice cultures, Purkinje cell dendritic outgrowth is regulated by granule cell inputs. © 2005 Elsevier B.V. All rights reserved.

Theme: Development and regeneration*Topic:* Process outgrowth, growth cones, and sprouting*Keywords:* NMDA-NR2 subunits; Organotypic slice cultures; Dendritic differentiation; Cerebellar granule cells; Purkinje cells

In cerebellar circuits, Purkinje cells play a central role for information processing, the tight relation between parallel fibre input and Purkinje cell morphology during development having an impact on motor performance in adulthood. Thereby, a stimulatory effect of granule cell synaptic activity has been shown where *N*-methyl-D-aspartate (NMDA) receptor (NR) activation positively modulated Purkinje cell dendrite outgrowth [8,19]. The former study further showed that this modulation is mediated by release of brain-derived neurotrophic factor (BDNF) from granule cells after NR stimulation, a concept supported by the fact that BDNF-deficient mice develop severely reduced Purkinje cell dendrites [17].

NRs mainly consist of NR1 and NR2A-2D subunits [9] which are expressed in a unique pattern in the cerebellum

[13,20]. Granule cells replace NR2B by NR2C during the first postnatal weeks coinciding with their migration and establishment of parallel fibre synapses [2,7]. This makes NR2C the predominant NR2 subunit in adulthood [3,4,9] and indicates a functional change of NR properties requiring NR2B for migratory granule cells to form and NR2C in mature granule cells to stabilise synapses. We recently described a knock-in mouse model (NR2C-2B mice) where sustained expression of NR2B under the NR2C promoter avoided this important subunit exchange [15]. Granule cell migration, survival and parallel fibre innervation were impaired in these mice suggesting impaired functionality of the parallel fibre-Purkinje cell synapse. Since input-dependent and NR-mediated mechanisms positively regulate Purkinje cell dendrite growth *in vitro* [8,12,19], we investigated Purkinje cell dendritic morphology in cerebellar slice cultures from NR2C-2B mice.

Mice with transgenic expression of NR2B under the NR2C promoter have been reported recently [15]. Experiments were performed in accordance with institutional and

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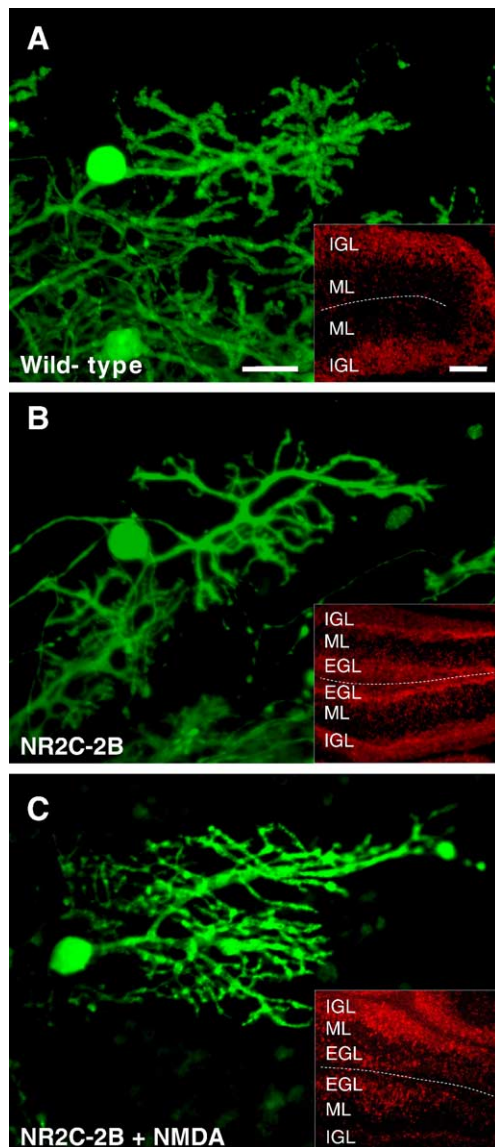


Fig. 1. Morphology of Purkinje cells in slice cultures from wild-type and NR2C-2B mice. Cerebellar slices were cultured for 12 DIV in the presence or absence of NMDA (200 μ M) and subsequently stained for Calbindin D-28K or NeuN. Shown are Purkinje cells and granule cells (NeuN stainings in the insets) from wild-type mice (A) or NR2C-2B mice grown in the absence (B) or presence of NMDA (C). For quantification, see Fig. 2. Note the presence of a prominent EGL in slices from NR2C-2B mice (insets in B, C). EGL, external granule cell layer; ML, molecular layer; IGL, internal granule cell layer; dotted lines delineate the cerebellar vermis of the folium. Scale bar in A, 30 μ m; inset, 100 μ m.

governmental ethical guidelines. Cerebellar slice cultures from 8 days old (P8) wild-type or NR2C-2B mice were prepared as described [6,16] and cultivated under serum-free conditions (Neurobasal with B27 and 2 mM glutamax I, all Gibco) on Millicell-CM membranes (Millipore) in a CO₂ incubator (37 °C). After incubation with or without NMDA (200 μ M, Tocris) for 12 days in vitro (DIV), slice cultures were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer. Rabbit Anti-calbindin (1:2000, Swant) and anti-NeuN (1:1000, Chemicon) immunostaining was visualised

with Cy2 and Cy3 labelled goat secondary antibodies (1:500, Jackson). Calbindin stained slice cultures were categorised into three classes according to number and distribution of Purkinje cells as described [5,16] to yield a semi-quantitative measure for Purkinje cell survival. Morphological analysis included measurement of dendritic length, area and number of branching points from calbindin stained Purkinje cells as described recently [18]. A correlation analysis between the area and the number of branching points per dendrite was fitted by linear regression and yielded the branching density (slope of regression line). Individual experiments with 6 to 10 slices per sample were

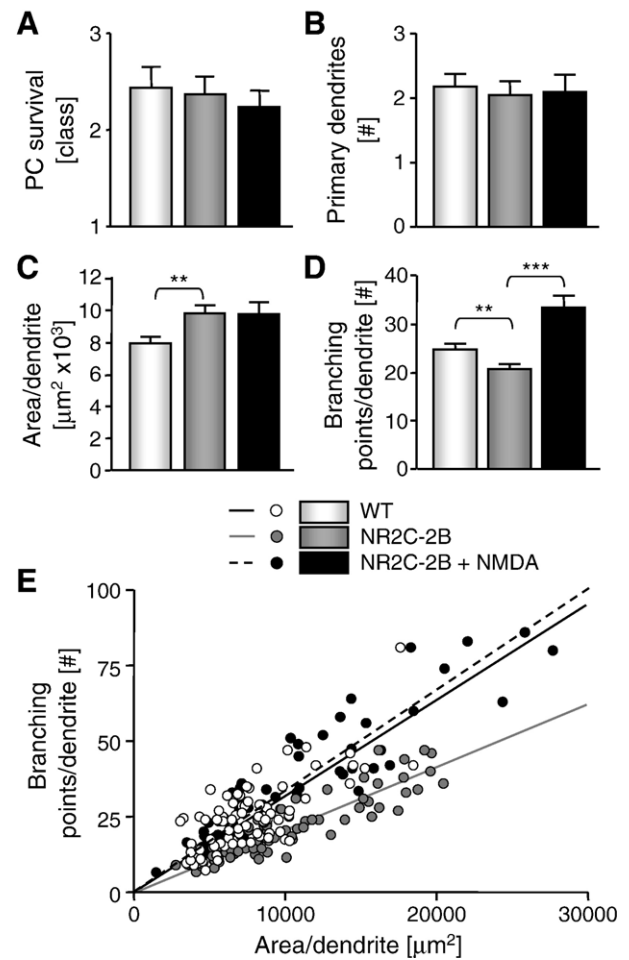


Fig. 2. Quantitative analysis of Purkinje cell survival and dendritic morphology in slice cultures from wild-type and NR2C-2B mice. Survival and morphological parameters were assessed from cultures of wild-type or NR2C-2B mice after 12 DIV. (A) Purkinje cell (PC) survival according to classification criteria ($n = 32$, 4 experiments). (B) Primary dendrites per Purkinje cell. (C) Area per individual dendrite. (D) Branching points per individual dendrite. (E) Correlation analysis between area and branching points/dendrite. The slopes of the regression lines for wild-type (black solid, $R = 0.716$), NR2C-2B (grey solid, $R = 0.908$) and NR2C-2B (black dashed, $R = 0.925$) cells yield the branching density (shown in Table 1). Data represent means \pm 95% CI from 32 slices (A) and 64–84 single Purkinje cells (B–E) from 4 independent experiments. ** $P < 0.01$; *** $P < 0.001$ as revealed by non-parametric Kruskal–Wallis ANOVA followed by Dunn's multiple comparison test.

repeated four times using matched controls yielding a total number of 64–84 single Purkinje cells analysed, and data were pooled. Results were expressed as means \pm 95% confidence interval (CI). Statistical significance of differences was assessed by non-parametric Kruskal–Wallis ANOVA followed by Dunn's multiple comparison test of the three experimental groups using GraphPad software (San Diego) considering differences significant for $P < 0.05$.

Under control conditions (Fig. 1A), wild-type Purkinje cells over 12 DIV developed a well-described dendritic phenotype [1,6,16] and NeuN staining revealed an expected granule cell layering consisting of a molecular layer (ML) and an internal granule layer (IGL). Purkinje cells from NR2C-2B mice developed seemingly underdeveloped dendrites with fewer branches but without apparent alterations in overall dendrite outgrowth (Fig. 2B). These cultures in contrast to wild-type cultures maintained an external granule layer (EGL) after 12 DIV (compare to [15]). NMDA treatment of NR2C-2B Purkinje cells resulted in an obvious recovery of this reduced phenotype (Fig. 1C) without apparent effect on number or distribution of granule cells.

Semi-quantitative evaluation of Purkinje cell survival was performed at DIV 12 and yielded similar classes for slices from wild-type and NR2C-2B mice with or without NMDA treatment (Fig. 2A, Table 1). Purkinje cell morphology analysis showed that the number of primary dendrites did not differ as well between the three experimental groups (Fig. 2B, Table 1). In contrast, NR2C-2B Purkinje cells developed with a slightly increased dendritic area ($P < 0.01$ vs. wild-type); NMDA treatment in these NR2C-2B cultures was without effect on this measure (Fig. 2C, Table 1). Furthermore, NR2C-2B Purkinje cells had fewer branching points than wild-types ($P < 0.01$) and NMDA treatment strongly increased this measure above wild-type levels ($P < 0.001$ vs. untreated NR2C-2B, Fig. 2D, Table 1). The branching density calculated as slope of the correlation between area and branches per dendrite was strongly reduced by $>30\%$ in untreated NR2C-2B Purkinje cells ($P < 0.001$ vs. wild-type) and fully recovered by NMDA treatment to wild-type levels ($P < 0.001$ vs. untreated NR2C-2B, Fig. 2E, Table 1).

In this study, we investigated whether the blockade of an exchange of the NR2 subunit naturally occurring in granule cells has any impact on Purkinje cell morphology in a well-characterised slice culture model. We observed that continuing NR2B expression instead of NR2C upregulation impairs Purkinje cell dendrite branching, and this impairment can be reversed by NMDA treatment of the mutant slices. These data are well in line with earlier reports demonstrating a direct correlation between NR function and dendritic growth in different neuronal populations including Purkinje cells [8,10,14,19,22] (reviewed in Refs. [11,21]).

The NR2C-2B mouse model that we used has recently been described [15]. In addition to endogenous NR2B, these mice strongly expressed NR2B from the mutant gene locus instead of NR2C already at P7, resulting in altered granular NR channel conductance indicating the functional presence of NR2B. In vivo, this resulted in a reduced ML size and IGL granule cell density already at P14–16. Moreover, adult NR2C-2B mice had 30% less parallel fibre inputs compared to wild-types. Slice culture data further provided evidence that granule cell migration was impaired [15]. Whether these morphological alterations are due to impaired or increased NR function was tested by treatment of the cultures with NMDA which increased Purkinje cell dendritic outgrowth. Our observations therefore support the concept that impaired granule cell maturation leads to reduced Purkinje cell dendritic branching probably as a result of less parallel fibres innervating the dendrites. This deficit may have been compensated or overcome by stimulation of the remaining granule cells and parallel fibres in NR2C-2B cultures with NMDA since a recovery of dendritic branching was observed. Such a positive modulation of Purkinje cell dendritic development by NR's has been shown in chick embryos where chronic treatment with an NR antagonist in ovo resulted in a reduction of the dendritic tree [19] and in cocultures with granule cells where NR activation stimulated and NR inhibition reduced Purkinje cell dendrite growth [8]. Thus, we can speculate that the genetic alteration in NR2C-2B mice most probably impaired NR dependent signaling. From our data, we cannot distinguish between a direct effect in Purkinje cells and an indirect

Table 1

Quantitative analysis of Purkinje cell survival and dendritic morphology in cerebellar slice cultures from wild-type and NR2C-2B mice

| | Wild-type | NR2C-2B | NR2C-2B + NMDA |
|--|-----------------|------------------------------|------------------------------|
| Purkinje cell survival (Class) | 2.44 \pm 0.22 | 2.38 \pm 0.18 | 2.25 \pm 0.16 |
| Primary dendrites (#/cell) | 2.2 \pm 0.2 | 2.1 \pm 0.2 | 2.1 \pm 0.3 |
| Area/dendrite (μm^2) | 7916 \pm 858 | 9768 \pm 992 ^a | 9775 \pm 1476 |
| Branching points/dendrite (#) | 24.7 \pm 2.5 | 20.7 \pm 2.2 ^a | 33.5 \pm 4.9 ^b |
| Branching density/dendrite (# $\times 10^{-3}$) | 3.18 \pm 0.11 | 2.08 \pm 0.04 ^c | 3.34 \pm 0.08 ^b |
| R for correlation analysis | 0.739 | 0.908 | 0.925 |

Purkinje cells from wild-type and NR2C-2B mice were cultured for 12 DIV with or without NMDA (200 μM). Data are expressed as mean \pm 95% CI (Purkinje cell survival, $n = 32$; other data, $n = 64$ –84 from each 4 experiments).

^a $P < 0.01$ vs. wild-type.

^b $P < 0.001$ vs. untreated NR2C-2B.

^c $P < 0.001$ vs. wild-type.

effect by alteration of granule cell maturation or function. Further work has to address this interesting issue.

Taken together, we have shown that the blockade of the cerebellar NR2B replacement by NR2C during the early postnatal period has a profound influence on Purkinje cell dendritic growth in vitro suggesting an important role of this receptor exchange for the development of proper cerebellar circuitry and motor function later in life.

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